

Assay of Colistin and Colistin Methanesulfonate in Plasma and Urine by Liquid Chromatography-Tandem Mass Spectrometry[▽]

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A rapid high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was developed for the routine quantification of colistins A and B and their prodrugs, colistin methanesulfonate (CMS) A and CMS B, respectively, in human plasma and urine by using polymyxin B1 as the internal standard (IS). CMS concentrations were determined indirectly by subtracting the colistin concentrations determined in biological samples from the whole colistin concentrations determined after sample treatment with sulfuric acid in order to hydrolyze CMS into colistin. After extraction on a solid-phase extraction column, the colistins were separated on an XBrigde C₁₈ column with isocratic elution (run time, 3.8 min). The mobile phase was 0.1% (vol/vol) formic acid in acetonitrile–0.1% (vol/vol) formic acid in water (20:80, vol/vol), run at a 0.2-ml/min flow rate. Ions were detected in the turbo-ion-spray-positive and multiple-reaction-monitoring modes. The ions monitored (precursor [M + 2H]²⁺ to product ions) were *m/z* 585.5/101.2 for colistin A, *m/z* 578.5/101.2 for colistin B, and *m/z* 602.5/241.2 for IS. Prevalidation studies demonstrated the stability of CMS in biological samples and extracts, a key point for the reliable quantification of colistin and CMS. The assay was accurate and reproducible for the quantification of colistins A and B and CMSs A and B in plasma samples over concentration ranges appropriate for pharmacokinetic studies: 0.024 to 6.144, 0.015 to 3.856, 0.029 to 7.492, and 0.010 to 2.508 µg/ml, respectively. In urine samples, the assay was validated over the same concentration ranges for colistins and over concentration ranges of 0.058 to 7.492 µg/ml and 0.020 to 2.508 µg/ml for CMSs A and B, respectively.

After being abandoned in the early 1980s because of reported nephrotoxicity and neurotoxicity (3), colistin is having a second life as a salvage treatment in critically ill patients, since it is often the last line of defense against multidrug-resistant Gram-negative bacteria, such as *Pseudomonas* spp. and *Acinetobacter* spp. (6, 15). Optimal dosing with colistin suffers from poor pharmacokinetic characterization, in part due to the challenge raised by assay of its properties when it is in biological fluids. Colistin is composed of at least 30 polymyxins, with the main fractions being colistin A (polymyxin E1) and colistin B (polymyxin E2), which account for more than 85% of colistin by weight (2, 14). It is available as a sulfate salt, colistin sulfate, used orally in bowel sterilization regimens, and as a methanesulfonated inactive (1) prodrug, so-called colistimethate or colistin methanesulfonate (CMS), used as a sodium salt in parenteral and aerosol dosage forms for systemic and local treatment, respectively. The prodrug, which itself is a complex mixture of methanesulfonated colistin derivatives, mainly CMS A and CMS B, is hydrolyzed into a series of partially methanesulfonated derivatives plus colistin *in vivo* (12). Investigation of the pharmacokinetic behaviors of CMSs A and B and colistins A and B is therefore necessary to set up guidelines for the dosing of patients. High-pressure liquid chromatography (HPLC) methods sensitive enough for pharmacokinetic studies of colistin require complex derivatization procedures (5, 7, 8).

Combining the quantification of colistin alone with the quantification of the whole colistin content (i.e., colistin plus methanesulfonated derivatives) permitted the determination of both the colistin and the methanesulfonated derivative concentrations in plasma samples (8). However, these HPLC methods are time-consuming (chromatographic run times are at least 15 min), and derivatization may lack interlaboratory reproducibility. Ma et al. (13) developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with electrospray ionization (ESI) and detection in the positive-ion mode for the quantification of colistins A and B in plasma and urine with short run times (less than 2 min). However, their method, which involved the pretreatment of biological samples with acid, was not validated in the presence of CMS, a crucial point to be investigated, considering that CMS, which is likely present in biological samples at early times postdosing, was reported to convert rapidly into colistin in various aqueous media and under acidic conditions (8, 9). More recently, Jansson et al. (4) solved this issue and proposed a LC-MS/MS method with ESI, but with detection in the negative-ion mode, for the quantification of colistins A and B and CMSs A and B by applying the two-step determination procedure described by Li et al. (8). This method was recently applied to an investigation of the pharmacokinetics of colistin and CMS in patients treated with intravenous CMS (16). Although this was a step forward in the development of an assay for determination of the concentrations of colistin and its prodrug in plasma, the chromatographic conditions dictated by the poorly efficient sample purification method applied resulted in 10-min run times. Given the limited stability of CMS in the samples

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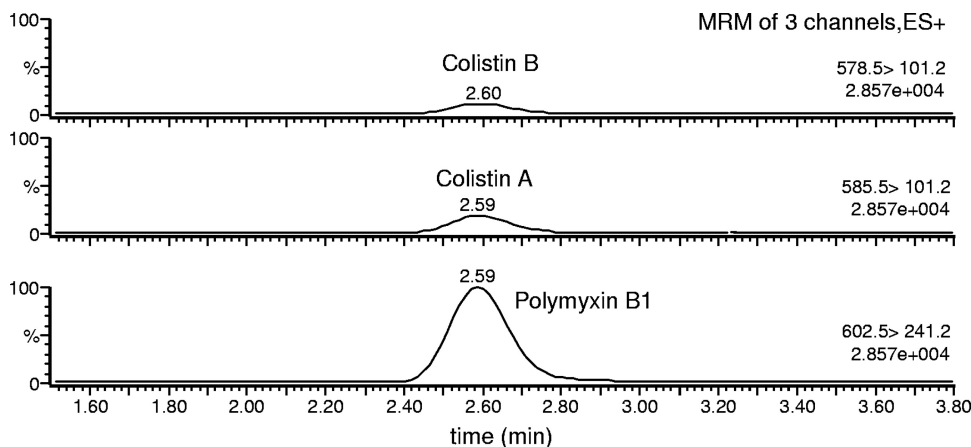


FIG. 1. Typical LC-MS/MS chromatograms obtained from a colistin calibration standard at the LLOQ. The plasma sample was spiked with $0.039 \mu\text{g} \cdot \text{ml}^{-1}$ of colistin, i.e., colistins A and B at concentrations of 0.024 and $0.015 \mu\text{g}/\text{ml}$, respectively (concentrations as the free bases), and $0.625 \mu\text{g} \cdot \text{ml}^{-1}$ of polymyxin B as the IS (concentration as free base). For details about the sample treatment, see the text.

worked up, each analytical sequence should not exceed 5 h (4), which corresponds to the time required for the analysis of 30 samples in the case of 10-min run times. Considering that in routine analytical practice 14 runs are devoted to standards ($n = 8$) and quality control (QC) samples ($n = 6$) and that a typical pharmacokinetic study comprises the analysis of samples obtained at 10 to 15 time points per individual, the analytical sequence allows the analysis of the pharmacokinetic profile of only one individual. The present work was therefore aimed at developing a LC-MS/MS assay for determination of the quantities of colistins A and B and CMSs A and B in plasma and urine with short chromatographic run times in order to increase the productivity of the analytical method. During the development of this method, the authors addressed the issue of the reference standards of these multicomponent drugs and prodrugs and of their stabilities in biological samples during storage, during sample pretreatment, and after extraction.

MATERIALS AND METHODS

Chemicals. The colistin sulfate salt and CMS sodium salt (100% purity, according to the provider's specification) used as standards, polymyxin B sulfate salt, methanol, formic acid (98 to 100% purity), acetonitrile, 10 M sodium hydroxide solution, and sulfuric acid (98 to 100% purity) were purchased from Sigma. Colimycin for injection (CMS sodium salt), administered to a healthy volunteer, was obtained from Sanofi-Aventis. Oasis HLB solid-phase extraction (SPE) cartridges (1 ml, 30 mg) were from Waters. All the solvents were of HPLC grade and were used without further purification. Highly purified water was produced by using a Millipore Milli-Q gradient plus system.

Plasma and urine samples. Human blank plasma was obtained from the Etablissement Français du Sang (EFS). Human urine was obtained from healthy drug-free volunteers. The results obtained for a volunteer participating in a pharmacokinetic study conducted at the University Hospital of Poitiers (Poitiers, France), after the receipt of approval by the local ethics committee (Region Poitou-Charentes CCPRB, protocol no. 05.12.26), are presented as an illustration of the application of this new assay. The volunteer received 80 mg of CMS as a 60-min intravenous infusion. Blood samples were collected in heparinized tubes, immediately chilled, and centrifuged. The plasma samples were stored at -20°C . Urine was collected over 24 h and was stored at -20°C .

Stock solutions and working standards. Stock solutions ($1,000 \mu\text{g} \cdot \text{ml}^{-1}$) of colistin (with the concentration given as that of the colistin base, i.e., without the sulfate moiety), CMS (with the concentration given as that of CMS, i.e., without the sodium ion), and the internal standard (IS; polymyxin B; with the concentration given as that of the polymyxin B base) were prepared in water. Working

solutions (concentration ranges, 0.039 to $5 \mu\text{g} \cdot \text{ml}^{-1}$ and $10 \mu\text{g} \cdot \text{ml}^{-1}$ for standards of colistin and CMS, respectively; $6.25 \mu\text{g} \cdot \text{ml}^{-1}$ for the IS) were obtained by appropriate dilution of independent (including a separate weighing of the analytical reference substances) stock solutions in water. The stock and working solutions were stored at -20°C .

Sample preparation for colistin and CMS LC/MS-MS determination. (i) **Calibration standards, validation standards (controls), and blanks.** Calibration standards (seven levels) and controls (three levels) were prepared in 5-ml polypropylene tubes, as follows. For the colistin calibration standards and controls, $250 \mu\text{l}$ of drug-free human plasma or $200 \mu\text{l}$ of drug-free urine supplemented with $100 \mu\text{l}$ drug-free plasma (to avoid the loss of colistin by adsorption) were spiked with $25 \mu\text{l}$ of the colistin working solutions and $25 \mu\text{l}$ of the IS working solution and then brought to 1 ml with water and briefly vortex mixed. The mixtures were loaded onto SPE columns preconditioned with 1 ml methanol followed by 2 ml water. After the columns were washed with 1 ml water, the analytes were eluted with 1 ml of 0.1% (vol/vol) formic acid in methanol. The eluates were then evaporated to dryness under a gentle nitrogen stream in a heating block at 45°C , and the residues were redissolved in $150 \mu\text{l}$ of formic acid 0.1% (vol/vol) in water and analyzed by LC-MS/MS. For the CMS calibration standards and controls, CMS was converted into colistin by acidic hydrolysis prior to the purification step. Thus, $250 \mu\text{l}$ of drug-free human plasma or $200 \mu\text{l}$ of drug-free urine supplemented with $100 \mu\text{l}$ drug-free plasma was spiked with $25 \mu\text{l}$ of the CMS working solutions and $25 \mu\text{l}$ of the IS working solution, and then the samples were acidified with $40 \mu\text{l}$ of 0.5 M sulfuric acid in water. After 1 h at room temperature, $40 \mu\text{l}$ of 1 M sodium hydroxide aqueous solution was added in order to neutralize the acidity and the volume was brought to 1 ml with water. The extraction procedure was conducted as described above for the colistin calibration standards. Blanks were prepared similarly, except that the colistin or CMS working solution was replaced with $25 \mu\text{l}$ water.

(ii) **Clinical samples.** Clinical samples were divided into two aliquots of $250 \mu\text{l}$ each for plasma and $200 \mu\text{l}$ each for urine in 5-ml polypropylene tubes. Each of the sample types was then treated by the procedure described above for the calibration standards and then analyzed by LC-MS/MS.

LC-MS/MS procedure. (i) **Apparatus.** The LC-MS/MS system consisted of a Waters Alliance 2695 separations module equipped with a binary pump and an autosampler thermostatically controlled at 4°C and a Waters Micromass Quattro micro-API triple-quadrupole tandem mass spectrometer.

(ii) **Chromatographic conditions.** Reversed-phase chromatography was performed on a C_{18} XBridge column ($5.0 \mu\text{m}$, 150 by 2.1 mm [inner diameter]; Waters). The mobile phase was 0.1% (vol/vol) formic acid in acetonitrile–0.1% (vol/vol) formic acid in water (20:80, vol/vol). The flow rate was $0.2 \text{ ml} \cdot \text{min}^{-1}$, and the injection volume was $20 \mu\text{l}$.

(iii) **MS-MS conditions.** The mass spectrometer was operated in the positive-ion mode. Ions were analyzed by multiple-reaction monitoring (MRM) by employing the transition of the $[\text{M} + 2\text{H}]^{2+}$ precursor to the product ions for the analytes and IS. The transition ions were m/z 585.5/101.2 for colistin A, m/z 578.5/101.2 for colistin B, and m/z 602.5/241.2 for the IS (polymyxin B1 [13]). They were found to be the dominant species, in agreement with the findings of

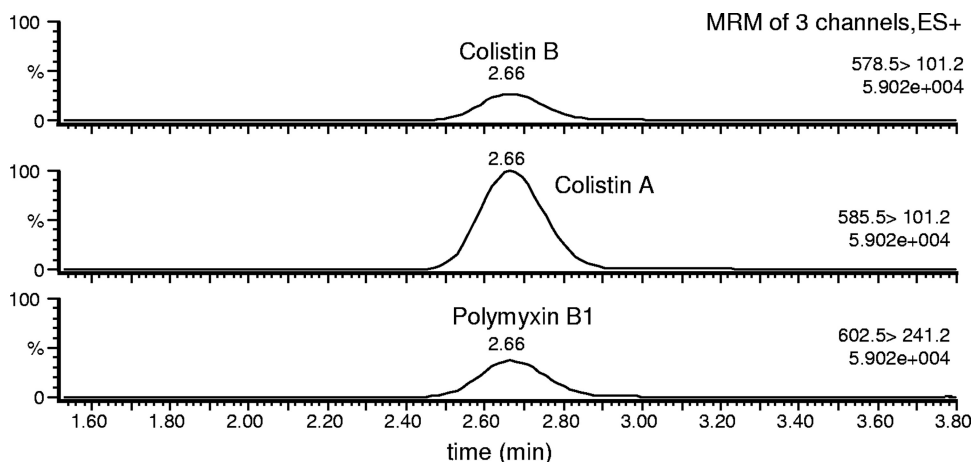


FIG. 2. Typical LC-MS/MS chromatograms obtained from the plasma sample of a healthy volunteer dosed with 80 mg CMS intravenously. The sample was spiked with $0.625 \mu\text{g} \cdot \text{mL}^{-1}$ of polymyxin B as the IS (concentration as the free base) and was analyzed for its colistin A and B contents. The concentrations of colistins A and B (as the free bases) were calculated to be 0.467 and 0.119 $\mu\text{g}/\text{mL}$, respectively.

previous studies (9, 17). The parameters were optimized by infusing $10\text{-}\mu\text{g}/\text{mL}$ solutions of colistin or polymyxin B into the LC-MS/MS interface by using a built-in syringe pump set at a flow rate of $10 \mu\text{L} \cdot \text{min}^{-1}$ in order to achieve the best signal-to-noise (S/N) ratio for the A and B subcomponents and the IS. The optimal MS/MS setup parameters were a $+3.0\text{-kV}$ ion spray voltage; a $600\text{-liter} \cdot \text{h}^{-1}$ and 350°C desolvation gas (N_2) flow and temperature, respectively; a $20 \text{ liter} \cdot \text{h}^{-1}$ cone gas (N_2) flow; a 120°C source temperature; a 35-V cone potential for colistin and the IS; 35-V and 25-V collision energies for colistin and the IS, respectively; and a 250-ms dwell time.

Prevalidation studies. (i) Colistin purity estimation. The purity of the colistin sulfate salt was estimated by the method described by Ma et al. (13) by applying a validated HPLC-UV method, conducted as follows: 210-nm detection wavelength, C_{18} XTerra column ($5.0 \mu\text{m}$, 150 by 3.9 mm [inner diameter]; Waters), a mobile phase of 0.1% (vol/vol) formic acid in acetonitrile– 0.1% (vol/vol) formic acid in water ($11:89$, vol/vol) run at $0.6 \text{ mL} \cdot \text{min}^{-1}$, and a $20\text{-}\mu\text{L}$ injection volume. The purity (weight percent) was calculated by dividing the summed areas of the two peaks of colistins A and B by the total area of the colistin chromatogram (excluding the peaks also found in blank samples).

(ii) Subcomponent A and B molar fraction (percent) determinations for colistin and CMS. As no pure colistin A and B reference standards were available, colistin A and B molar fractions (percent) were estimated as follows. For the colistin sulfate salt, they were estimated from the relative areas of the peaks for

colistins A and B obtained by two chromatographic methods: the present LC-MS/MS method and the HPLC-UV method described above. As both methods gave consistent results with the colistin sulfate salt (which demonstrated the same extinction coefficient or ionization efficiency for both colistins A and B; see Results), only the LC-MS/MS method was applied to determine the molar concentrations of subcomponents A and B in the CMS standards after acidic hydrolysis into colistins A and B.

(iii) Selection of plasma sample pretreatment method. Two sample pretreatment methods were compared: one without an acidic deproteinization step (referred to as nonacidic pretreatment, which was further used in the present work; see above for details of the sample preparation) and one with a deproteinization step (referred to as acidic pretreatment) performed by methanolic trifluoroacetic acid (TFA) protein precipitation and based on the procedure described by Ma et al. (13). The latter was carried out as follows: CMS-spiked plasma samples ($200 \mu\text{L}$) were added to $800 \mu\text{L}$ of a $50:50$ (vol/vol) mixture of methanol– 10% (vol/vol) TFA solution in water; after the solutions were mixed and the mixture was centrifuged, the supernatants were purified on SPE columns as described above and the extracts were analyzed by LC-MS/MS.

(iv) Urine samples. When the method developed for determination of the colistin and CMS concentrations in plasma samples was directly applied to urine samples, the method lacked reproducibility. This problem, attributed to the adsorption of colistin onto the plasticware, was solved by supplementing the

TABLE 1. Stabilities of CMSs A and B in stock solutions, spiked plasma and urine samples, and plasma extracts under various conditions^a

Sample type	Conditions	Initial CMS A-CMS B concn ($\mu\text{g}/\text{mL}$)	Colistin A-colistin B concn determined ($\mu\text{g}/\text{mL}$) ^b	CMS A-CMS B degradation (mol%)
Stock solution	-20°C over 5 mo	749.2–250.8	No peak detected	No degradation
Spiked plasma samples	-20°C or -80°C over 2 mo	3.746–1.254	No peak detected	No degradation
	Nonacidic pretreatment	3.746–1.254	No peak detected	No degradation
	Acidic pretreatment (protein precipitation)	7.492–2.508	$1.626 \pm 0.141\text{--}0.485 \pm 0.041$	32.5–29.1
	Two freeze-thaw cycles	0.937–0.313	No peak detected	No degradation
	RT ^c for 1 h	3.746–1.254	$0.123 \pm 0.023\text{--}0.029 \pm 0.004$	3.3–2.3
	RT for 4 h	3.746–1.254	$0.519 \pm 0.074\text{--}0.175 \pm 0.019$	13.9–14.0
Plasma extracts	5 h at 4°C (in autosampler)	3.746–1.254	No peak detected	No degradation
Spiked urine samples	Two freeze-thaw cycles	3.746–1.254	$0.746 \pm 0.053\text{--}0.180 \pm 0.015$	19.9–14.4
	RT for 0 h	3.746–1.254	$0.614 \pm 0.027\text{--}0.143 \pm 0.003$	16.4–11.6
	RT for 1 h	3.746–1.254	$0.835 \pm 0.115\text{--}0.211 \pm 0.022$	22.3–16.8
	RT for 4 h	3.746–1.254	$1.056 \pm 0.006\text{--}0.268 \pm 0.022$	29.2–21.4

^a Colistin is the degradation product of CMS.

^b Values are means \pm SDs ($n = 5$).

^c RT, room temperature.

TABLE 2. Validation of method of determination of colistin A and B concentrations in plasma^a

Compound	Intraday							
	Validation				Calibration curve ^b			
	Accuracy (<i>n</i> = 6)			Precision (<i>n</i> = 6)	<i>a</i>	<i>b</i>	Intercept, <i>c</i>	<i>r</i> ²
	Nominal concn (μg/ml)	Determined concn (μg/ml)	Bias (%)					
Colistin A	0.024	0.026 ± 0.000	6.7	0.8	0.1438 ± 0.0569	4.4721 ± 0.2935	0.0358 ± 0.00477	0.9966 ± 0.0007
	0.384	0.378 ± 0.018	−1.6	4.8				
	6.144	5.939 ± 0.292	−3.3	4.9				
Colistin B	0.015	0.014 ± 0.002	−3.7	13.3	0.1729 ± 0.0398	3.7589 ± 0.4526	0.0045 ± 0.0003	0.9905 ± 0.0030
	0.241	0.241 ± 0.016	0	6.5				
	3.856	4.031 ± 0.351	4.5	8.7				

^a The LLOQs were 0.024 and 0.015 μg/ml for colistins A and B, respectively. Calibration data were fitted by quadratic least-squares regression ($y = ax^2 + bx + c$) of the peak area ratios (i.e., drug peak area/IS peak area = y) versus concentrations x , using $1/x^2$ as the weighting factor.

^b The values are means ± SDs ($n = 3$ for the intraday validation and $n = 4$ for the interday validation). The concentration ranges were 0.024 to 6.144 μg/ml for colistin A and 0.015 to 3.856 μg/ml for colistin B.

urine samples with blank human plasma (100 μl plasma to 200 μl urine sample), as previously described for determination of the colistin concentration in a cell culture medium (4). Urine samples supplemented with plasma were handled like the plasma samples.

Validation procedure. The validation procedure was applied to the separate quantification of subcomponents A and B of colistin and CMS.

(i) **Concentration units.** Final concentrations were expressed as μg · ml^{−1} of colistins A and B as the free bases or CMSs A and B without the sodium ion. Intermediate calculations required conversion into molar units by using 1,169.48, 1,155.45, 1,634.89, and 1,620.86 g · mol^{−1} as the molar masses of colistins A and B (free bases) and CMSs A and B, respectively.

(ii) **Calibration curves.** Seven-point calibration standard curves were established for subcomponents A and B of colistin and CMS over a concentration range covering the concentrations expected in patient plasma samples. The calibration data were fitted by quadratic least-squares regression ($y = ax^2 + bx + c$) of the peak area ratios (i.e., drug peak area/IS peak area = y) versus concentrations x , using $1/\text{concentration}^2$ ($1/x^2$) as the weighting factor. The weighting factor was selected by backcalculation of the concentrations and examination of the distribution of the residuals. The acceptance criteria were as follows: the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels, and at least two-thirds of the standards, including the highest and the lowest calibration levels, met these criteria.

(iii) **Accuracy.** The accuracy of the method was evaluated by using validation standards and was expressed as the percent error between the mean concentrations determined for the validation standards and their nominal concentrations against their nominal concentrations (percent bias; $n = 6$).

(iv) **Precision.** Precision was investigated by studying the intraday (repeatability) and interday (reproducibility, except for urine) variations in the concentrations determined for validation standards at three concentration levels taken from the calibration range ($n = 6$). The coefficients of variation (CVs; in percent) were calculated from the estimated concentrations.

(v) **LLOQ.** The LLOQ was defined as the lowest concentration providing satisfactory repeatability (i.e., CV < 20%) and bias (i.e., <20%). They were estimated for subcomponents A and B of colistin and CMS.

(vi) **Recoveries.** The extraction recoveries of the analytes from plasma and urine were calculated by comparing the responses of the extracted analytes to the responses of the IS-spiked blank plasma extracts spiked with the analytes (at the corresponding nominal concentrations).

Stability studies. The long-term stabilities were assessed at −20°C over 5 months for the colistin and CMS stock solutions (1,000 μg · ml^{−1}) and at −20°C and −80°C over 2 months for the spiked plasma samples (1.25 μg · ml^{−1} for colistin and 5 μg · ml^{−1} for CMS). Short-term stability studies, performed to define the sample-handling conditions, were carried out as follows. The stability of a colistin- or CMS-spiked plasma or urine sample was studied under two freeze-thaw cycles (1.25 μg · ml^{−1} for colistin and 1.25 μg · ml^{−1} [plasma] or 5 μg · ml^{−1} [urine] for CMS; temperature cycle, from −20°C to room temperature within 5 min and then down to −20°C in a freezer and maintenance at −20°C for 24 h before the second thawing was performed) and at room temperature for 1

or 4 h (1.25 μg · ml^{−1} for colistin and 5 μg · ml^{−1} for CMS). The stabilities of the extracts (obtained from plasma spiked with 1.25 μg · ml^{−1} for colistin and 5 μg · ml^{−1} for CMS) in an autosampler at +4°C were evaluated over 5 h.

RESULTS

LC-MS/MS chromatograms. The LC-MS/MS chromatogram of a colistin standard at the LLOQ is shown in Fig. 1. CMS standards hydrolyzed into colistin gave similar chromatograms (data not shown). The chromatographic peaks of colistins A and B and the IS had the same retention times (about 2.6 min). The peak superimposition was circumvented by the MS/MS detection of the analytes in three separate paired ion channels. The plasma sample from the healthy volunteer gave similar chromatographic profiles for determination of the colistin A and B concentrations (Fig. 2) and the whole colistin content (data not shown).

Prevalidation and stability studies. (i) **Colistin purity and colistin and CMS subcomponent A and B molar fractions.** The purity of the colistin sulfate salt used as the standard was determined to be 87% by HPLC-UV analysis. Virtually equal relative signal responses for subcomponents A and B of colistin were obtained by HPLC-UV analysis (61.5 and 38.5% for subcomponents A and B, respectively) and LC-MS/MS (62.7 and 37.3% for subcomponents A and B, respectively). Therefore, calculation of the molar fractions (in percent) from the responses obtained either by HPLC-UV analysis or by LC-MS/MS gave similar results. Accordingly, LC-MS/MS alone was applied to determine the molar fractions of the subcomponent A and B CMS compounds: 74.9 and 25.1% for subcomponents A and B, respectively, for CMS from Sigma and 79.2 and 20.8% for subcomponents A and B, respectively, for CMS from Sanofi-Aventis.

(ii) **Stability studies.** For colistin, long-term stability studies showed no significant degradation in stock solutions stored at −20°C for 5 months or in spiked plasma samples stored at −20°C and −80°C over a 2-month period (data not shown). No colistin degradation was observed under the following conditions: acidic or nonacidic plasma pretreatment, two freeze-thaw cycles (urine or plasma), storage of colistin-spiked plasma

TABLE 2—Continued

				Interday			
Validation				Calibration curve ^b			
Accuracy (<i>n</i> = 6)				<i>a</i>	<i>b</i>	Intercept, <i>c</i>	<i>r</i> ²
Nominal concn (μg/ml)	Determined concn (μg/ml)	Bias (%)	Precision (<i>n</i> = 6)				
0.048	0.047 ± 0.005	−1.6	10.1	−0.0305 ± 0.2226	5.0675 ± 0.3393	−0.0099 ± 0.2072	0.9961 ± 0.0028
0.192	0.178 ± 0.016	−7.4	8.9				
1.536	1.537 ± 0.156	0	10.2				
0.030	0.031 ± 0.003	3.7	11.2	−0.2970 ± 0.3038	5.3308 ± 0.2251	−0.0205 ± 0.0382	0.9977 ± 0.0010
0.120	0.113 ± 0.008	−6.7	7.2				
0.964	0.967 ± 0.098	0.3	10.1				

or urine samples at room temperature for 4 h, and storage of plasma sample extracts at 4°C in the autosampler for 5 h (data not shown).

For CMS, the long-term stability studies showed no significant degradation in stock solutions stored at −20°C for 5 months or in spiked plasma samples at −20°C and −80°C over a 2-month period (Table 1). Acidic pretreatment of CMS-spiked plasma samples resulted in significant hydrolysis into colistin, whereas no hydrolysis into colistin was observed after nonacidic pretreatment (Table 1). No CMS degradation was detected after two freeze-thaw cycles of the plasma samples or after storage of the extracts at +4°C in the autosampler for 5 h, but colistin A and B peaks were detected after 1 h (negligible) and 4 h (about 14% degradation, considered unacceptable) of storage of CMS-spiked plasma samples at room temperature. A degradation of 10 to 30% CMS into colistin was observed for CMS-spiked urine samples under the conditions tested (Table 1). It should be noted that conversion started virtually immediately when the urine samples were stored at room temperature.

Validation. Validation results are presented in Tables 2 and 3 (plasma) and Table 4 (urine).

(i) **LLOQs.** The LLOQs were close for subcomponents A and B either of colistin or of CMS in plasma. The LLOQs in urine were not determined, as the concentrations in clinical samples are relatively high.

(ii) **Calibration curves.** Satisfactory coefficients of determination ($r^2 > 0.99$) were obtained for the calibration curves for plasma (Tables 2 and 3) and urine (urine had the same concentration range as plasma for colistins A and B and concentration ranges of 0.058 to 7.492 μg/ml and 0.020 to 2.508 μg/ml for CMSs A and B, respectively; results not shown).

(iii) **Accuracy.** For both subcomponents A and B either of colistin or of CMS, the bias values for the validation standards (controls) never exceeded 15%, a usual acceptance criterion (Tables 2 and 3 for plasma and Table 4 for urine).

(iv) **Precision.** At the three concentrations studied, the precision was acceptable, with the CV values being less than 15% (Tables 2 and 3 for plasma and Table 4 for urine).

(v) **Recoveries.** For the plasma samples, extraction recoveries were 69.4% ± 2.3%, 72.9% ± 1.0%, and 66.6% ± 4.9% for colistins A and B and the IS, respectively. For the urine sam-

ples, they were 72.2% ± 9.1%, 77.2% ± 7.0%, and 66.4% ± 0.5% for colistins A and B and the IS, respectively.

Samples from healthy volunteer. Typical plasma concentration-versus-time curves for colistins A and B and CMSs A and B are presented in Fig. 3. The cumulative fractions of the dose recovered in urine as CMS and colistin over 24 h were determined to be 43 mol% and 18 mol% of the injected dose, respectively.

DISCUSSION

The aim of the present work was to develop a reliable and rapid analytical method for the quantification of colistin and CMS in human plasma and urine in order to provide reliable pharmacokinetic data after CMS administration. In setting up an analytical method for determination of the concentrations of chemically complex and variable compounds like colistin and its prodrug, CMS, particular attention should be given to the establishment of reference standards (11).

For the quantification of colistin in plasma, published LC analytical methods used the colistin sulfate salt as the standard and relied only on the two major chromatographic peaks (which corresponded to colistins A and B) (4, 5, 7, 13). Considering that colistins A and B represent at least 85 percent (by weight) of the raw material (2), which was confirmed in the present work (see Results), this practice is generally considered to be an acceptable approximation, with no other practical alternative being available (i.e., the lack of availability of individual standards of colistins A and B [13]). In order to quantify colistins A and B individually, the molar fractions (percent) of colistins A and B in the colistin sulfate batch, used as a reference, were estimated by HPLC-UV analysis, according to the method of Li et al. (7), and by the LC-MS/MS method described here. Although the two methods are based on two different signal generation techniques, they gave virtually equal relative signal responses for the two chromatographic peaks of subcomponents A and B (see Results), demonstrating the consistency of the procedure and showing that the UV extinction coefficients and MS/MS ionization efficiencies are similar between the two subcomponents. This is not surprising, since colistins A and B differ chemically by only one methylene residue of the fatty acid moiety, which is not in-

TABLE 3. Validation of method of determination of CMS A and B concentrations in plasma^a

Compound	Intraday							
	Validation				Calibration curve ^b			
	Accuracy (<i>n</i> = 6)			Precision (<i>n</i> = 6)	<i>a</i>	<i>b</i>	Intercept, <i>c</i>	<i>r</i> ²
	Nominal concn (μg/ml)	Determined concn (μg/ml)	Bias (%)					
CMS A	0.029	0.031 ± 0.002	8.6	6.7	−0.0491 ± 0.0219	2.3607 ± 0.0911	0.2848 ± 0.0075	0.9943 ± 0.0034
	1.873	2.058 ± 0.045	9.9	2.2				
	7.492	7.927 ± 0.262	5.8	3.3				
CMS B	0.010	0.009 ± 0.001	−9.5	6.1	−0.1470 ± 0.0653	2.1088 ± 0.0924	0.0830 ± 0.0039	0.9960 ± 0.0028
	0.627	0.666 ± 0.019	6.2	2.8				
	2.508	2.797 ± 0.087	11.5	3.1				

^a The LLOQs were 0.029 and 0.010 μg/ml for CMSs A and B, respectively. Calibration data were fitted by quadratic least-squares regression ($y = ax^2 + bx + c$) of the peak area ratios (i.e., drug peak area/IS peak area = *y*) versus concentrations *x*, using $1/x^2$ as the weighting factor.

^b The values are means ± SDs (*n* = 3 for the intraday validation and *n* = 4 for the interday validation). The concentration ranges were 0.029 to 7.492 μg/ml for CMS A and 0.010 to 2.508 μg/ml for CMS B.

volved in the UV absorption or ionization phenomenon. As determination of the colistin concentration in collected biological samples relies on the preservation of CMS, particular care was taken to study and to prevent the hydrolysis of CMS during plasma sample storage and handling and during sample pretreatment prior to the assay. Acidic pretreatment as a deproteinization procedure was found to result in significant CMS hydrolysis (Table 1). Therefore, for studies of the pharmacokinetics of colistin in patients treated with CMS, strongly acidic sample pretreatment, as was used by Ma et al. (13), should be avoided. Jansson et al. (4) controlled the hydrolysis of CMS by using a low acid concentration and by strictly controlling the time of exposure to acidic conditions, constraining those investigators to processing batches of 12 samples within 10 to 15 min. The mild conditions of sample pretreatment used in the present work (plasma sample dilution with water, followed by SPE, referred to as nonacidic pretreatment) also preserved CMS without such a constraint (Table 1). Stability studies gave further information on conditions for the storage and handling of colistin and/or CMS-containing samples: (i) in stock solutions, colistin and CMS were found to be stable at −20°C over 5 months; (ii) in spiked plasma samples, they were found to be stable over 2 months at −20°C and −80°C; (iii) two freeze-thaw cycles could be applied to the plasma samples; (iv) plasma samples should be treated within 1 h after they are thawed in order to preserve the CMS; and (v) in agreement with the findings of previous work (4), the extracts should be analyzed within 5 h when they are kept in an autosampler at 4°C (Table 1). Considering the large number of samples generated by pharmacokinetic studies (generally 10 to 15 per individual) and the stability of the extracts for 5 h during storage in autosamplers at +4°C, assay development was aimed at providing run times as short as possible for optimal analytical productivity. By using MS/MS detection, the physical separation of the analytes by the chromatographic column was not required, as MS/MS permitted their specific detection without compromising the analytical performance (13). This high degree of specificity, combined with the efficient purification of biological samples by SPE, which suppressed the matrix effect arising from sample impurities, allowed the chromatographic run

times to be as short as 3.8 min (Fig. 1). The method allows 75 determinations over the 5-h period of stability of extracts in an autosampler at 4°C, which, when the use of 14 standards and QC samples is considered, should permit the analysis of at least four pharmacokinetic profiles per analytical sequence, whereas the method of Jansson et al. (4) permitted the analysis of only one pharmacokinetic profile per analytical sequence. Furthermore, in order to correct for potential recovery and/or instrumental variations, an internal standard was included with the method. The use of such a precaution, proposed by Li et al. (who used netilmicin as the IS [7]) and Ma et al. (who used polymyxin B1 as the IS [13]), was not retained by Jansson et al. (4). Polymyxin B1 was preferred to netilmicin because of its chemical similarity to colistin (13). The method of determination of colistins A and B in plasma was validated over concentration ranges adequate for pharmacokinetic studies (Table 2), and the LLOQ was similar to that of Jansson et al. (4).

For determination of the CMS concentration in plasma, it was not realistic to determine the concentration of each substituted form of CMS, as CMS is a multimethanesulfonated colistin derivative that hydrolyzes into a series of less methanesulfonated derivatives *in vivo*. Therefore, like the work of Li et al. (8) and Jansson et al. (4), the present LC-MS/MS method of determination of the plasma CMS concentration was indirect and combined the concentrations of each substituted form of CMS. By subtracting the colistin (A and B) concentrations determined in biological samples by the method discussed above from the whole colistin concentrations determined after acidic hydrolysis, the CMS concentrations could be calculated. However, instead of using a calibration curve built from colistin standards, as in the previous work (4, 8), calibration curves were built from CMS standards hydrolyzed into colistin by acidic pretreatment. Although it complicated the procedure, pretreatment of the calibration standards in the same way as the true samples was considered to minimize methodological errors. The method of determination of the plasma CMS concentration was validated over concentration ranges adequate for pharmacokinetic studies (Table 3), confirming the reliability of the hydrolytic conversion of CMS into colistin.

TABLE 3—Continued

Validation				Interday ^c			
Accuracy (<i>n</i> = 6)				Calibration curve ^b			
Nominal concn (μg/ml)	Determined concn (μg/ml)	Bias (%)	Precision (<i>n</i> = 6)	<i>a</i>	<i>b</i>	Intercept, <i>c</i>	<i>r</i> ²
0.059	0.056 ± 0.003	−4.8	6.0	−0.1154 ± 0.2457	5.4850 ± 0.4123	0.1640 ± 0.0161	0.9942 ± 0.0030
0.234	0.250 ± 0.011	6.7	4.3				
1.873	2.037 ± 0.198	8.8	9.7				
0.020	0.020 ± 0.002	−1.7	11.0	0.0344 ± 0.3368	5.2261 ± 0.4517	0.1640 ± 0.0161	0.9925 ± 0.0061
0.078	0.084 ± 0.006	6.9	6.9				
0.627	0.700 ± 0.089	11.6	12.7				

For the determination of colistin and CMS concentrations in urine, the same procedure used for the determination of the concentrations in plasma was basically applied. Urine samples were supplemented with blank human plasma in order to minimize the adsorption of the colistin onto the plasticware (4). In urine, the hydrolysis of CMS into colistin was found to be significant at room temperature and to start immediately after the urine was spiked (Table 1); this is also likely to occur in the bladder and/or in urine collection containers. This uncontrolled hydrolysis could lead to the underestimation of CMS urinary excretion and to the overestimation of colistin urinary excretion, as documented previously (10).

The method was applied in a pharmacokinetic study with a healthy volunteer dosed with CMS intravenously (3a). Each biological sample was split into two aliquots and analyzed according to two independent assays. One aliquot was assayed for determination of the colistin A and B concentrations (resulting from CMS conversion *in vivo*) by using the colistin sulfate salt as the calibration standard. The second aliquot, treated with sulfuric acid in order to hydrolyze CMSs A and B

into colistins A and B, respectively, was assayed for determination of the whole colistin A and B contents, using CMS standards that were similarly treated, and the CMS concentrations were calculated. The results of the plasma pharmacokinetics are presented in Fig. 3. In plasma, the CMS A concentrations were severalfold higher than those of CMS B, but both decayed in parallel. The same observation was made for colistins A and B. As expected, plasma CMS peak concentrations were higher and occurred earlier than those of colistin, but the disappearance of the prodrug was relatively fast and the CMS concentrations became lower than those of colistin after about 3 h postdosing (Fig. 3). In urine, the cumulative fractions of the dose recovered as CMS and colistin over 24 h were found to be 43 mol% and 18 mol%, respectively. However, considering the likely postexcretion conversion of CMS into colistin (as discussed above), the fraction values may be underestimated in the case of CMS and overestimated in the case of colistin.

TABLE 4. Intraday validation of methods of determination of colistin A and B and CMS A and B concentrations in urine^a

Compound	Accuracy (<i>n</i> = 6)			Precision (<i>n</i> = 6) ^b
	Nominal concn (μg/ml)	Determined concn (μg/ml)	Bias (%)	
Colistin A	0.024	0.024 ± 0.004	−1.6	14.9
	0.384	0.367 ± 0.015	−4.5	4.2
	6.144	5.623 ± 0.419	−8.5	7.5
Colistin B	0.015	0.015 ± 0.001	−3.9	8.7
	0.241	0.239 ± 0.019	−0.9	8.0
	3.856	3.548 ± 0.274	−8.0	7.7
CMS A	0.058	0.058 ± 0.002	0.7	3.7
	1.873	1.973 ± 0.242	5.3	12.3
	7.492	8.154 ± 0.229	8.8	2.7
CMS B	0.020	0.020 ± 0.003	1.2	12.5
	0.627	0.714 ± 0.073	13.9	10.2
	2.508	2.653 ± 0.217	5.8	8.2

^a Calibration data were fitted by quadratic least-square regression ($y = ax^2 + bx + c$) of the peak area ratios (i.e., drug peak area/IS peak area = *y*) versus concentrations *x*, using $1/x^2$ as the weighting factor.

^b Precision is presented as repeatability (percent CV).

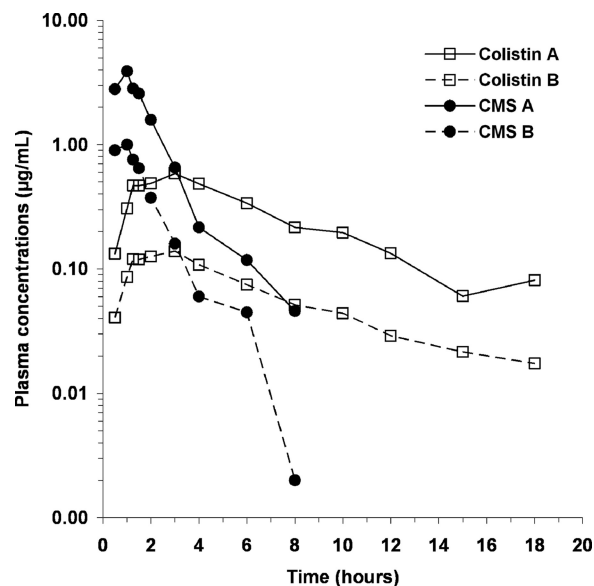


FIG. 3. Concentrations of colistins A and B (concentrations as the free base) and CMSs A and B (concentrations as colistin methanesulfonate, i.e., without sodium ion) versus time in plasma from a typical volunteer dosed with 80 mg CMS as a 60-min intravenous infusion.

In conclusion, the present LC-MS/MS method with controlled sample cleaning allows rapid analysis (run times, 3.8 min). Such a short run time permits 75 analyses to be performed over the 5-h period of stability of the sample extracts in an autosampler at +4°C. This should permit reliable and sensitive determinations of the pharmacokinetic parameters of colistins A and B and CMSs A and B in patients intravenously treated with CMS. The CMS concentrations in urine, however, should be interpreted with care, as these are likely to be underestimated due to a likely rapid and significant postexcretion conversion of CMS into colistin.

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